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Optimization of the human factor VIII complementary DNA expression plasmid for gene therapy of hemophilia A

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While the gene delivery vehicle is critical for the efficacy of human factor VIII gene therapy, optimization of the potency and duration of the factor VIII gene that is delivered is equally important in light of the poor transcription and translation characteristics of this gene. We discuss here a systematic approach to optimization of factor VIII complementary DNA expression by analysis of specific elements engineered into the transcription unit and other positions in the expression plasmid. Within the transcription unit we have engineered different 5' and 3' sequence modifications and tested them for factor VIII expression in human liver cells. These changes incorporate liver-specific promoter and enhancer sequences and regulatory elements affecting RNA export. Specifically, the thyroid hormone-binding globulin promoter and α_1 microglobulin/bikunin enhancer were tested and a synthetic 5' intron was compared to a 3' post-transcriptional regulatory element on factor VIII expression levels. For translation optimization, a leader sequence was designed to be of optimum length, have no RNA secondary structure and contain the optimal translation initiation sequence. Finally, we discuss areas for plasmid optimization, which include removal of near-consensus splicing sequences, the inclusion of strong transcription termination elements and the use of autonomous replicating plasmid sequences for episomal maintenance and enhanced plasmid retention for duration of gene expression.

Keywords: factor VIII complementary DNA, transcription, gene therapy

Introduction

The next generation of therapy for patients with hemophilia A is being developed as a ligand-targeted gene complex which enters a liver hepatocyte and delivers its genetic payload. The delivery of functional factor VIII genes to liver hepatocytes and their prolonged expression at therapeutic levels will abrogate the necessity for daily factor VIII injections by patients for prophylaxis. To this end, the delivery vehicle for targeting the gene to its ultimate location in the nucleus of the hepatocyte and the gene itself require extensive optimization. Here we describe our strategy for optimization of factor VIII complementary (c)DNA expression plasmids and present comparative expression studies with various examples of these gene constructs.

Cloning of human factor VIII cDNA was reported in the early 1980s [1–4] and since then both gene and protein have been extensively studied because of their importance to clinical medicine. The full length factor VIII cDNA was observed to express at very low levels [5] relative to engineered versions where the B domain was excised from the cDNA [6]. The B domain of human factor VIII protein is not required for procoagulent activity [7,8] and is normally proteolysed to varying degrees during secretion from the cell and circulation in plasma. While factor VIII post-translational processing and trafficking through the cell may be responsible in a large part for its poor secretion levels (relative to similar coagulation proteins such as factor V) [9,10], the factor VIII RNA itself appears to be poorly transported from nucleus to cytoplasm. It has been reported that as much as 90% of the RNA does not exit the nucleus and is thus degraded. Studies have shown that retroviral titers are 100-fold lower when retroviruses are engineered to contain the B domain-deleted version

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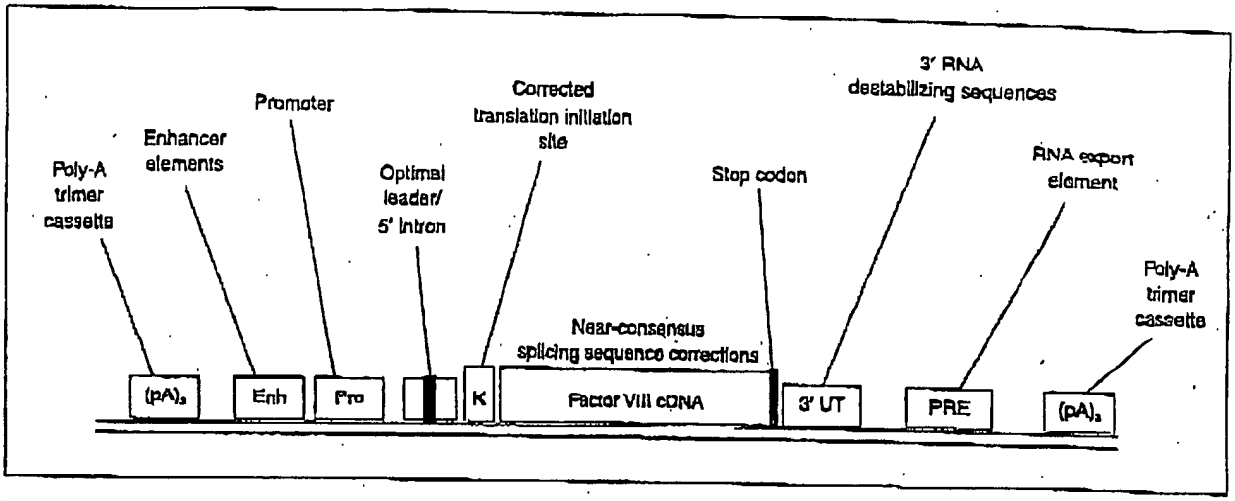

Fig. 1. Proposed areas within the human factor VIII transcription unit for sequence optimization. cDNA, complementary DNA; UT, untranslated; PRE, post-transcriptional regulatory element.

ered as candidates for transcriptional activity. The potent enhancer sequence from the α_1-microglobulin/bikunin gene, described by Rouet *et al.* [16], and the liver-specific promoter sequence from the thyroxine-binding globulin gene described by Hayashi *et al.* [14], were selected as the promoter/enhancer pair for these studies. The α_1-microglobulin/bikunin enhancer contains a cluster of hepatic nuclear factor elements common to many liver-specific genes within a short DNA sequence, making it ideal to multimerize. When multimerized, this enhancer, like many others, shows incremental activity and functions in either orientation in the gene [16]. The thyroxine-binding globulin promoter was chosen because of its tissue-specific activity and the presence of hepatic nuclear factor enhancer elements as well as the precise mapping of its transcriptional start site [14]. These characteristics allowed us to insert a leader sequence designed to contain no RNA secondary structure, as predetermined by an RNA-folding algorithm (Fig. 2). Minimizing secondary structure in a leader sequence is important, since this has been associated with increased translation potential [15].

To complete the 5' untranslated region of our transcription unit (identified in Fig. 1 and shown in Fig. 2), we made two additional modifications. First, we changed the DNA sequence at the translational start site to make it optimal for translation initiation [17]. Human factor VIII normally contains a cytosine at the +4 position following the AUG start codon, which was changed to a guanine. This base change resulted in an amino acid change in the factor VIII signal sequence from a glutamine to a glutamic acid. An optimal translation initiation sequence may be critical since 10 in-frame AUG codons, some of which fit the consensus sequence for transcriptional initiation, occur downstream of the first AUG codon [5]. Second, we introduced an optimized intron into the leader sequence for efficient access of the RNA into the splicing/nucleocytoplasmic export pathway. The cDNAs, being generated by reverse transcription of messenger (m)RNA, are naturally devoid of introns and, relative to their genomic counterparts, are not expressed by the cell as efficiently. The inclusion of an intron in a cDNA transcription unit is known to increase mRNA levels [19], probably as the result of the tight coupling between RNA splicing and export to the cytoplasm. Therefore we chose intron 1 from the rabbit β-globin gene to include in the transcription unit but altered it to contain a perfectly consensus 5' splice donor sequence (CAGGTA AGT) and replaced five purines with pyrimidines within the existing pyrimidine tract (Fig. 2).

We investigated the influence of one and two copies of the liver-specific α_1-microglobulin/bikunin enhancer and inclusion of the 5' intron in a sequential manner on factor VIII expression *in vitro* (Table 1). The liver-specific mammalian 5' regulatory region was also compared to the human cytomegalovirus enhancer/promoter regulatory region. The results clearly showed that the presence of two copies of the α_1-microglobulin/bikunin enhancer element had the greatest effect on expression level, resulting in an overall 18-fold increase in expression. The presence of the 5' intron increased expression 3.5-fold, resulting in a combined effect of over 60-fold in active factor VIII expression. The viral cytomegalovirus promoter/enhancer remained the strongest 5' region, however, and resulted in an addi-

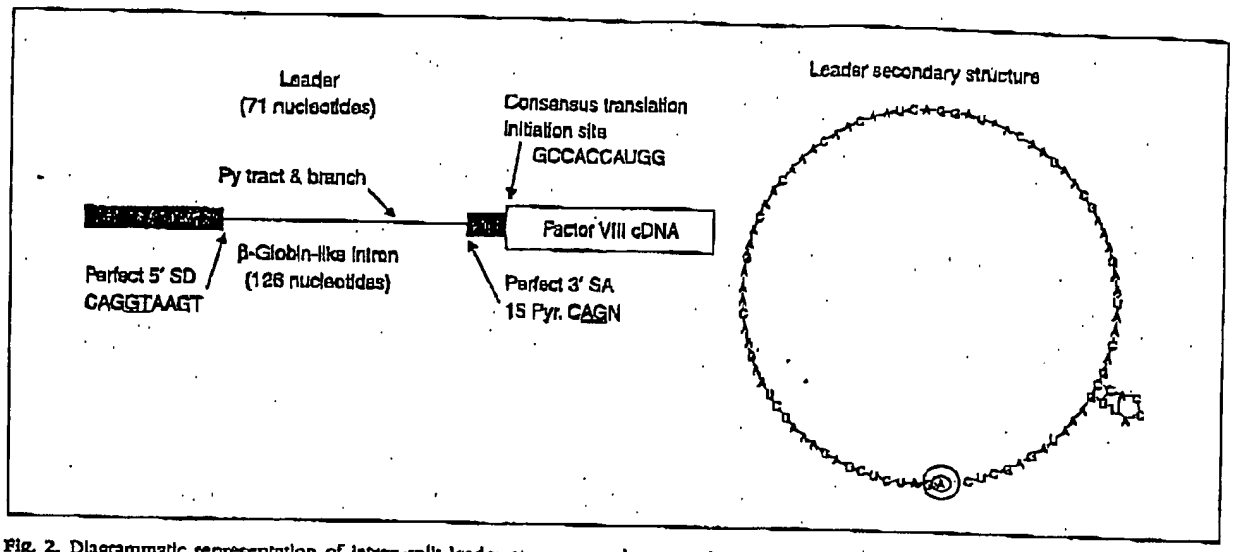

Fig. 2. Diagrammatic representation of intron-split leader sequence and proposed secondary structure. Details of the engineered intron are described in the text. The secondary structure of the leader sequence was predicted by the program RNAdraw obtained through the Internet. cDNA, complementary DNA.

Table 1. Influence of 5' regulatory element or intron on factor VIII expression levels *in vitro*

Specific 5' region tested	Fold increase
TBG promoter only	1
TBG promoter, 5' intron	3.5
One-copy ABP enhancer, TBG promoter, 5' intron	8.6
Two-copy ABP enhancer, TBG promoter, 5' intron	2.1
CMV immediate-early promoter only	2.3

TBG, thyroid hormone-binding globulin; ABP, α_1-microglobulin/bikunin; CMV, human cytomegalovirus.

tional 2.3-fold increase in factor VIII expression compared with the mammalian liver-specific region. However, as discussed below, the viral cytomegalovirus promoter/enhancer regulatory region does not appear to be useful for *in vivo* gene therapy applications.

Figure 3 shows a computer analysis of the presence of near-consensus 5'-splice donor sites and 3'-splice acceptor sites throughout the human B domain-deleted factor VIII cDNA. The abundance of these near-consensus splicing sites throughout the cDNA is striking. Seventeen of the 5'-splice donor sequences are within or preceed the putative transcriptional silencer and inhibitory sequences as defined by Hoeben *et al.* [13] and Lynch *et al.* [11], while 18 of the 23 splice acceptor sequences (with pyrimidine tracts) are within or follow these regions (shaded box regions, Fig. 3). These potential splicing sequences may play a role in the suboptimal expression levels of this gene in an analogous manner to the aberrant splicing recently shown for the low expression of human factor IX in the mammary gland of transgenic sheep [22]. Because factor VIII RNA nuclear export is known to be inefficient, we hypothesized that these potential splice sites could be partially responsible for low factor VIII expression by interaction with spliceosome complexes.

We designed two approaches for addressing the potential inhibitory effects of these postulated sites. The first strategy was to eliminate these sites from the cDNA sequence by mutation of either GT in the splice donor or AG in the splice acceptor site, respectively. In most cases, the redundancy of the genetic code permits a single base mutation in each site to functionally eliminate the site without changing the amino acid. Additionally, exploiting current knowledge of exon definition and the optimal size limitations of internal exons, as described by the Berget group [23], specific 3'-splice acceptor sites become more likely problematic. Such an analysis would, in theory, narrow down the mutagenesis effort needed.

While this approach is currently under way in our laboratory, we have investigated a second approach to augment factor VIII RNA export from the nucleus, which focuses on a viral element, termed the post-transcriptional regulatory element [24]. This is an element from the hepatitis B virus which is present in the 3' end of each of the viral genomic and subgenomic RNA species [20,24]. The post-transcriptional regulatory element acts in *cis*, using host cellular factors, and facilitates the cytoplasmic accumulation of these RNAs via a non-splicing RNA export pathway (Fig. 4). We have shown previously that this element will facilitate factor VIII expression, as measured by mRNA and pro-

tein, to a greater extent than a 3' intron from simian virus (SV40) [21]. When one or two copies of the post-transcriptional regulatory element are included at the 3' end of the factor VIII gene between the stop codon and polyadenylation signal, significant increases in expression are seen *in vitro* compared to no post-transcriptional regulatory element or the absence of a 5' intron in the construct. One or two copies of post-transcriptional regulatory element showed an additional twofold or fivefold increase in factor VIII expression, respectively (Fig. 5). However, compared to constructs with a 5' intron, a two-copy post-transcriptional regulatory element-containing gene did not impart as high an increase in factor VIII expression as did the upstream intron (Fig. 5). Currently we are testing other post-transcriptional regulatory-like elements, specifically the constitutive transport element found in type D retroviruses, for potency differences in their ability to facilitate the transport of factor VIII RNA and prevent unwanted 'aberrant' splicing.

There are additional modifications to consider at the 3' UTR of the transcription unit for optimal RNA processing, export and mRNA stability. Translational termination efficiency in mammalian RNAs is optimal if the base following the stop codon is a purine [25]. This is the case in the human factor VIII cDNA, where the UGA stop codon is followed by a guanine. Human factor VIII cDNA contains three pentamer sequences

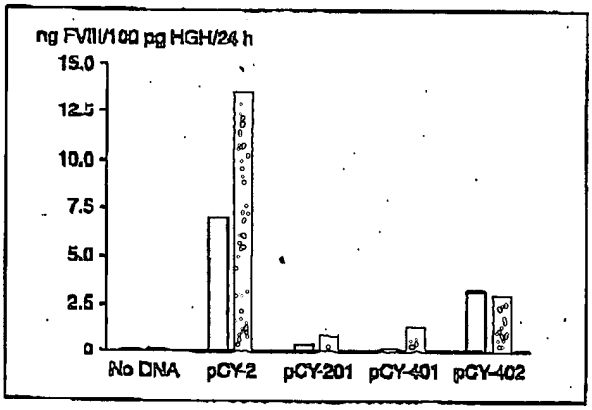

Fig. 5. Effect of a 5' intron and 3' post-transcriptional regulatory element on human factor VIII (FVIII) expression levels. HuH-7 cells were transfected with plasmid pCY 2, 201, 401 or 402 along with plasmid cytomegalovirus–human growth hormone (pCMV–HGH). Plasmids were constructed and transfections carried out as described in Materials and methods. The levels of secreted active factor VIII was measured from supernatants collected 48 h (first bar of each group) or 72 h (second bar of each group) after transfection by Coatest VIII:c/4 kit from Chromogenix AB, Inc. The transfection efficiency of each plasmid was normalized by analysis of human growth hormone secreted levels.

of AUUUA in the 3' UTR. While these sites have been shown to be destabilizing to RNAs, a larger nonamer sequence has been implicated [26] which is not present in the factor VIII 3' UTR. We have not investigated whether removal of these pentamer sequences will affect the message stability of factor VIII since removal of these sequences from the 3' UTR location does not always lead to stabilization of a message [27] (H.C. Chiou, unpublished observations, 1997). Switching the 3' UTR of a short-lived mRNA for one that has a longer lifespan may impart a half-life difference and is being investigated in our studies. The fate of an mRNA, however, is ultimately determined by elements located throughout the transcript and many sequence elements may be involved in mRNA decay. Sequences that confer stability on another mRNA have not yet been elucidated [27]. As mentioned above, we are examining elements in the 3' UTR that include viral elements for accessing non-splicing RNA export pathways as well as the poly A trimer [14] for optimal polyadenylation and 3'-end formation (Fig. 1).

Finally, within the plasmid but outside the transcription unit, sequence elements can be engineered into the plasmid to impart both autonomous replication activity (when the cell replicates its chromosomes the plasmid replicates as well) and nuclear retention as an episome. The best characterized sequence elements which confer these properties on a plasmid are the oriP sequence and EBNA-1 gene from the Epstein–Barr virus [28]. Unfortunately, however, the use of the Epstein–Barr virus sequences in vectors for gene therapy are confounded by the fact that they are active in human cells but not in rodent cells. This species-specific activity precludes preclinical gene therapy studies in mice and rats from using plasmids containing these elements. Instead, the problem has been addressed by the addition of large (16–20 kb) human genomic DNA sequences which impart an origin of replication activity to the plasmid and function in both rodent and human cells [29,30]. While there is concern that such sequences could potentiate transformation in human cells, there is no direct evidence for this occurrence. The fact that plasmids containing these sequences are maintained episomally may minimize any insertional mutagenesis due to random integration while their autonomous replication could ultimately decrease the number of gene transfers required [31]. It is clear from *in vitro* studies [30] that vectors containing these sequences show prolonged gene expression in a variety of mammalian cells.

Discussion

We have presented our strategy and results to date for optimization of the human factor VIII cDNA expression plasmid for future human gene therapy protocols.

While our present analysis and data set are not complete, certain engineered changes have already shown significant effects on improved expression of this complex gene.

Our rationale for liver-specific regulation of factor VIII expression was based on several considerations. First, the gene delivery vehicle we are developing will target the factor VIII gene via a ligand specific for the asialoglycoprotein receptor found on the liver hepatocytes, the cells which normally produce factor VIII. Second, though viral promoter/enhancer regulatory regions are commonly used for high-level cDNA expression, we have observed attenuated duration of factor VIII expression as well as that of other genes *in vivo*, with the viral cytomegalovirus promoter/enhancer relative to liver-specific constructs (data not shown). The reason(s) for attenuated duration of expression *in vivo* by these viral-driven genes is unknown; however, the recent elucidation of immunostimulatory sequences within cytomegalovirus sequences may be one area for future investigation [32]. Lastly, there are many secretory proteins from the liver for which genes have been well characterized with respect to tissue-specific regulation and are readily available for testing.

Enhancers have long been known to function in an orientation- and location-independent manner to markedly increase gene expression. Interestingly, enhancers may increase the probability of a construct being active and not the number of transcripts generated as a result of their presence in the gene [33]. Introns, however, have been shown to increase levels of mRNA as a result of the transcript efficiently accessing the splicing pathway, which is functionally coupled to RNA export. In the above studies, it was the combination of these two elements which resulted in the greatest increase in expression of the factor VIII cDNA.

The poor expression levels of cDNAs relative to their intron-containing genomic counterparts has long been recognized [19]. Studies have suggested that the problem appears to be due to reduced intranuclear stability and inefficient export of the transcripts from the nucleus to the cytoplasm [34,35]. Because RNA splicing, polyadenylation and nuclear export are tightly coupled processes in higher eukaryotes, the addition of intronic sequences in either a 5' or 3' location relative to the cDNA in the expression plasmid has been used to improve expression levels [36–38]. While intron addition facilitates higher mRNA levels in most cases, aberrant splicing is known to occur as a result of the spliceosome complex using near-consensus splice sites within the cDNA itself [39]. Optimal cDNA expression may require introns which are efficiently spliced and hence preferentially chosen over potential splice sites within the cDNA. Alternatively, inclusion of elements such as the post-transcriptional regulatory element may prevent (or reverse) the entry of the pre-mRNA into the splicing pathway, and hence allow its export from the nucleus. Our working hypothesis is that the abundance of near-consensus splice sites in factor VIII cDNA may be involved in the poor nucleocytoplasmic transport of this RNA. This hypothesis is supported by our analysis of the overlap of these sites with known transcriptional inhibitory regions in the cDNA (Fig. 3) [11–13]. Removal of these potential splice sites or accessing an alternate RNA export pathway may be a necessary requirement for optimal *in vivo* expression of factor VIII as well as other cDNAs.

The potential for insertional mutagenesis will remain a concern for patients undergoing gene therapy protocols. Optimization of cDNA gene expression level and duration, as described here, is critical since it will purport to require less frequent administration and lower dosing. Human tissue-specific regulation of the therapeutic gene and efficient mRNA nuclear export will result in high-level prolonged expression of the transferred gene in the desired tissue. Likewise, plasmid sequences which confer episomal nuclear maintenance and autonomous replication may be desirable but will require appropriate clinical trials before they can be deemed safe.

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